Available online at www.sciencedirect.com

ScienceDirect

Virology 364 (2007) 169–177

VIROLOGY

www.elsevier.com/locate/yviro

Nucleotides within the anticodon stem are important for optimal use of tRNA^{Lys,3} as the primer for HIV-1 reverse transcription

Anna McCulley, Casey D. Morrow *

Department of Cell Biology, University of Alabama at Birmingham, 802 Kaul Building, 720 20th Street, South Birmingham, AL 35294-0024, USA

Received 20 December 2006; returned to author for revision 18 January 2007; accepted 9 February 2007
Available online 21 March 2007

Abstract

HIV-1 utilizes tRNA^{Lys,3} as the primer for initiation of reverse transcription. To further examine the tRNA sequence and structural requirements for primer selection, we developed a complementation system which required tRNA^{Lys} to be provided *in trans*. We constructed an HIV-1 provirus in which the primer-binding site (PBS) was altered to be complementary to the 3′ terminal 18-nucleotides of *E. coli* tRNA^{Lys,3}, which shares many bases with mammalian tRNA^{Lys,3}, and demonstrated that infectious virus was obtained only if the provirus was co-transfected with the plasmid encoding *E. coli* tRNA^{Lys,3}. In the current study we have mutated *E. coli* tRNA^{Lys,3} so that nucleotides within the stem of the anticodon stem–loop were made identical to mammalian tRNA^{Lys,3}. Analysis of the complementation revealed that the modified *E. coli* tRNA^{Lys,3} (*E. coli* tRNA^{Lys,3}-MA) complemented 3–5 times more efficiently than *E. coli* tRNA^{Lys,3}. Mutation of nucleotides within the anticodon stem region of *E. coli* tRNA^{Lys,3}-MA that differed from *E. coli* tRNA^{Lys,3} revealed the importance of the nucleotide sequence for efficient use in reverse transcription. The results of our studies highlight that multiple regions of mammalian tRNA^{Lys,3} are important for the preference of tRNA^{Lys,3} as the primer for HIV-1 reverse transcription.

Keywords: HIV; tRNA; Primer binding site; Reverse transcription

Introduction

Human immunodeficiency type 1 (HIV-1), like most lentiviruses, exclusively selects tRNA^{Lys,3} as the primer for reverse transcription (Mak et al., 1997; Marquet et al., 1995). Previous studies from this laboratory and others have shown that HIV-1 has the capacity to select alternative tRNAs as the primers for reverse transcription (Das et al., 1995; Li et al., 1994; Wakefield et al., 1995). Alteration of the PBS to be complementary to numerous tRNAs allows HIV-1 to utilize these tRNAs for replication. However, the viruses rapidly revert to utilize tRNA^{Lys,3} as the primer for reverse transcription unless additional mutations are made upstream of the PBS in a region called the A-loop, which is complementary to the anticodon of tRNA^{Lys,3} (Kang and Morrow, 1999; Kang et al., 1997, 1999; Wakefield et al., 1996; Zhang et al., 1998). Additional regions within the HIV-1 genome have also been found to potentially be important in primer selection and reverse transcription. A region (primer activation signal) complementary to the TΨC stem–loop (nucleotides 50–57) of tRNA^{Lys,3} is located upstream of the PBS in the HIV-1 genome (Beerens et al., 2001). tRNA^{Lys,3} also plays a role in the second strand transfer during completion of the minus-strand DNA (Brule et al., 2000). Interaction between tRNA^{Lys,3} and U3 during the first strand transfer of HIV reverse transcription is facilitated by complementarity between the U3 and nucleotides within the stem of the tRNA^{Lys,3} anticodon RNA stem–loop.

To further understand the mechanism of primer selection, it would be advantageous to be able to manipulate the tRNA primer used for reverse transcription. Since it is difficult to effectively manipulate endogenous levels of tRNA in mammalian cells, previous studies from this laboratory have described a complementation system in which an exogenous tRNA, yeast tRNA^{Phe}, was supplied *in trans* to HIV-1 which had the PBS mutated to be complementary to the 3′ terminal 18-nucleotides

* Corresponding author. Fax: +1 205 934 5733.
E-mail address: caseym@uab.edu (C.D. Morrow).

0042-6822/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2007.02.010

of yeast tRNA^{Phe} (Kelly and Morrow, 2003, 2005; Yu and Morrow, 2000, 2001). Expression of yeast tRNA^{Phe} from a cDNA resulted in a tRNA that undergoes aminoacylation, nuclear transport, and inclusion into the host cell protein synthesis cycle (Kelly and Morrow, 2005). Using a defined set of mutants, we were able to ascertain that transport from the nucleus to the cytoplasm is a key requirement for the tRNA to be selected as a primer for HIV-1 reverse transcription (Kelly et al., 2003). Since previous studies have suggested that the reason for the preferred selection of tRNA^{Lys,3} as a primer is due to the incorporation of lysyl-tRNA synthetase into HIV-1 virions, it was important to develop a system which more accurately recapitulated the HIV-1 primer selection process and demonstrated a preference for tRNA^{Lys,3} (Cen et al., 2002; Javanbakht et al., 2003). To achieve this goal, we engineered an HIV-1 with a PBS complementary to *E. coli* tRNA^{Lys,3}, which maintains many of the similar features of mammalian tRNA^{Lys,3} (McCulley and Morrow, 2006). Using this system, we were able to demonstrate that HIV-1 replication could be complemented *in trans* with *E. coli* tRNA^{Lys,3} supplied in the form of a cDNA. Analysis of *E. coli* tRNA^{Lys,3} anticodon mutants revealed no correlation between aminoacylation and the ability to complement, indicating that interactions with the lysyl-tRNA synthetase do not fully explain the preferential usage of tRNA^{Lys,3} as a primer (McCulley and Morrow, 2006).

The level of complementation observed using *E. coli* tRNA^{Lys,3} was lower to that observed for the yeast tRNA^{Phe} system, indicating no preferential use of *E. coli* tRNA^{Lys,3} even though this tRNA interacts with lysyl-tRNA synthetase. To further explore this result in the current study, we have mutated the cDNA encoding *E. coli* tRNA^{Lys,3} to be identical to mammalian tRNA^{Lys,3} with the exception of the acceptor stem and the necessary 3′ terminal 18-nucleotides that anneal with the PBS. We have compared the complementation of *E. coli* tRNA^{Lys,3} and the modified *E. coli* tRNA^{Lys,3} (tRNA^{Lys,3}-MA). Results of our studies demonstrate that tRNA^{Lys,3}-MA was approximately 3 to 5-fold more efficient in complementation than *E. coli* tRNA^{Lys,3} due to nucleotides within the

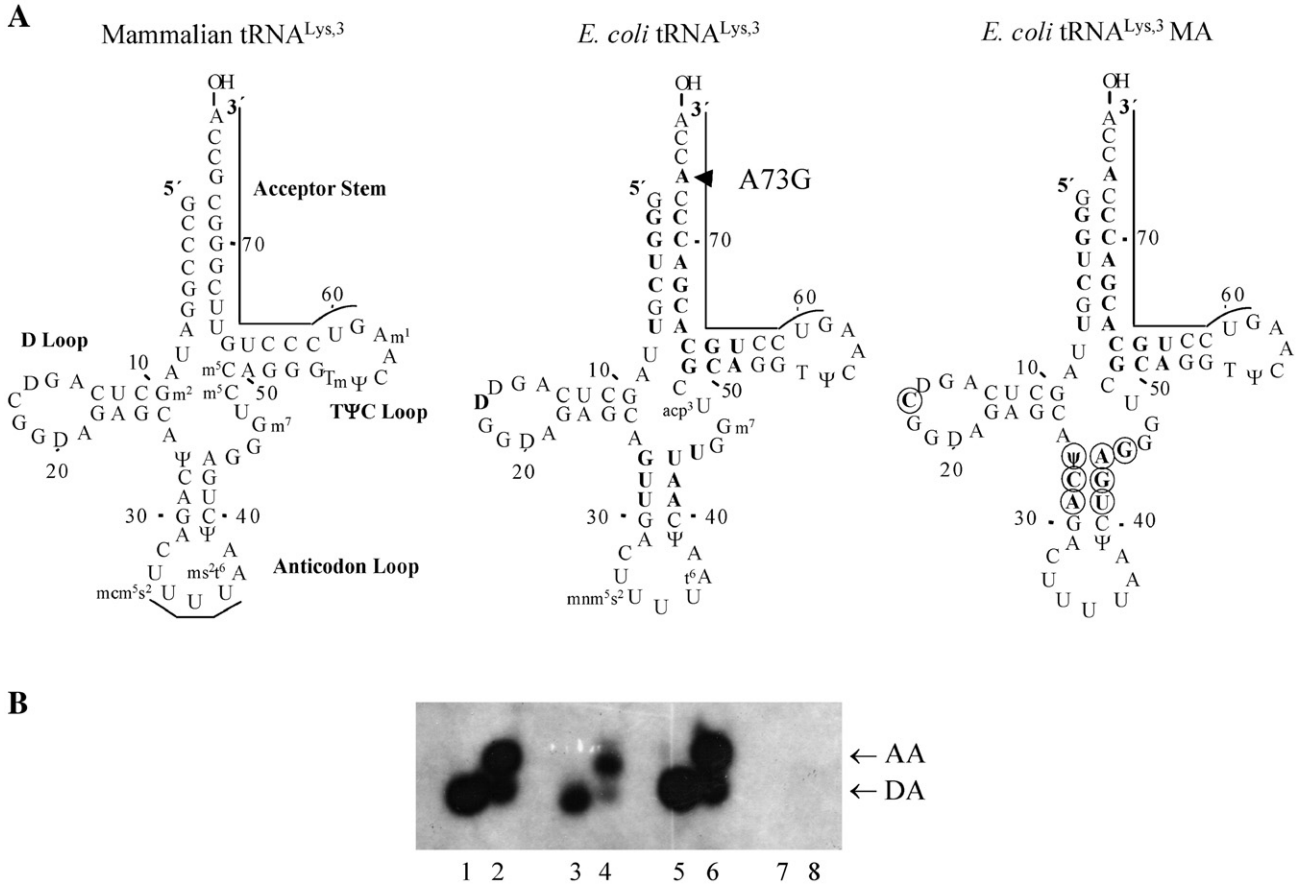

Fig. 1. Mammalian tRNA^{Lys,3}, *E. coli* tRNA^{Lys,3}, and *E. coli* tRNA^{Lys,3}-MA. (A) The cloverleaf depiction of mammalian tRNA^{Lys,3}, *E. coli* tRNA^{Lys,3}, and *E. coli* tRNA^{Lys,3}-MA. The 3′ terminal 18-nucleotides and the anticodon region are outlined in the mammalian tRNA^{Lys,3}. The boldface nucleotides in the *E. coli* tRNA^{Lys,3} represent base variance between mammalian and *E. coli* tRNA^{Lys,3} sequence. The A73G mutation is indicated by an arrow. Circled nucleotides in the *E. coli* tRNA^{Lys,3}-MA represent nucleotides that have been changed from *E. coli* tRNA^{Lys,3} sequence to mammalian tRNA^{Lys,3} sequence. (B) Aminoacylation of *E. coli* tRNA^{Lys,3}, *E. coli* tRNA^{Lys,3}-MA, and *E. coli* tRNA^{Lys,3}(A73G). Migration of the aminoacylated (AA) and deacylated (DA) samples is shown. Cytoplasmic tRNAs were collected from 293HEK cells that were transfected with pU6*Ec*^{Lys}, pU6*Ec*^{Lys}(A73G), and pU6*Ec*^{Lys}(MA). All cytoplasmic RNA was isolated under acidic conditions. Lanes 1 and 2 were loaded with cytoplasmic RNA from pU6*Ec*^{Lys}(A73G) transfection; lanes 7 and 8 were loaded with cytoplasmic RNA from mock transfection; lanes 5 and 6 were loaded with cytoplasmic RNA from pU6*Ec*^{Lys} transfection; lanes 3 and 4 were loaded with cytoplasmic RNA from pU6*Ec*^{Lys}(MA) transfection. All lanes were probed for *E. coli* tRNA^{Lys,3}. DA controls were prepared by pH adjustment (basic) and incubation for 1 h at 42 °C. DA samples are shown in lanes 1, 3, 5, and 7.

anticodon stem and the variable loop of *E. coli* $tRNA^{Lys,3}$. The results of these studies are discussed with respect to the preferential selection of $tRNA^{Lys,3}$ as the primer for HIV-1 reverse transcription.

Results

In a previous study, we described the construction and characterization of a complementation system in which *E. coli* $tRNA^{Lys,3}$ was provided *in trans* to an HIV-1 genome in which the PBS was altered to be complementary to the 3′ terminal 18-nucleotides of *E. coli* $tRNA^{Lys,3}$ (McCulley and Morrow, 2006). While we demonstrated complementation with respect to production of infectious virus, the level of complementation was not substantially higher than that found for a similar system which utilized yeast $tRNA^{Phe}$ and an HIV-1 proviral genome with the PBS complementary to 3′ terminal 18-nucleotides of yeast $tRNA^{Phe}$. Thus, we have not recapitulated the enhanced preference of HIV-1 for $tRNA^{Lys,3}$. Comparison of mammalian $tRNA^{Lys,3}$ with *E. coli* $tRNA^{Lys,3}$ revealed considerable changes within the anticodon stem region between the two tRNAs. To determine if alteration of these anticodon stem nucleotides could enhance the capacity of *E. coli* $tRNA^{Lys,3}$ to complement the HIV-1 viral genome, we constructed a hybrid *E. coli* $tRNA^{Lys,3}$ (*E. coli* $tRNA^{Lys,3}$-MA) that consisted of *E. coli* $tRNA^{Lys,3}$ components (acceptor stem, TΨC stem–loop) and mammalian $tRNA^{Lys,3}$ components (variable loop, anticodon stem–loop, D stem–loop) (Fig. 1A).

The first step in the characterization of *E. coli* $tRNA^{Lys,3}$-MA was to determine if it undergoes aminoacylation as *E. coli* $tRNA^{Lys,3}$. Following transfection, RNA was isolated under acidic conditions to maintain the integrity of the amino acid–tRNA bond. The samples were analyzed by gel electrophoresis and probed for *E. coli* $tRNA^{Lys,3}$. A deaminoacylated control was obtained by treating a portion of the sample with high pH buffer to break the amino acid–tRNA bond. We created a second *E. coli* $tRNA^{Lys,3}$ mutant which contained an A73G mutation so that the last four nucleotides of *E. coli* $tRNA^{Lys,3}$ would be identical to the last four nucleotides of mammalian $tRNA^{Lys,3}$ (Fig. 1A). The nucleotide at position 73 has also been implicated as a discriminator base (Huang et al., 1996; Shiba et al., 1997). Analysis of the samples revealed that all tRNAs were predominately aminoacylated following expression in mammalian cells (Fig. 1B). Slight differences were observed that could be attributed to processing given that the amino acid–tRNA bond is highly heat and pH sensitive. No significant differences were observed in the intracellular expression of the tRNAs using a range of plasmid concentrations (data not shown).

Complementation of HIV genomes containing a PBS complementary to E. coli $tRNA^{Lys,3}$

We first compared the capacity of *E. coli* $tRNA^{Lys,3}$ and *E. coli* $tRNA^{Lys,3}$-MA to complement the HIV-1 proviral genome containing a PBS complementary to *E. coli* $tRNA^{Lys,3}$ (Fig. 2A). Using a fixed ratio of tRNA plasmids to proviral plasmids, we generated virus and tested it for infectivity. The amount of infectious virus produced is determined following infection of JC53-BL cells, which encode a luciferase gene positioned downstream from an HIV-1 LTR. Following infection, the expression of Tat results in the transcription of luciferase, which is then detected using a standard assay. Previous studies have demonstrated that the amount of luciferase produced in the standard assay correlates with the amount of infectious virus (McCulley and Morrow, 2006; Wei et al., 2002). In our complementation system, the production of infectious virus was dependent upon the co-transfection of the proviral genome and plasmids encoding *E. coli* $tRNA^{Lys,3}$. Under the standard conditions, the amount of infectious virus produced by co-transfection with *E. coli* $tRNA^{Lys,3}$-MA was approximately 3–5 times to that produced by co-transfection with *E. coli* $tRNA^{Lys,3}$ (Fig. 2B). The amount of virus produced by co-transfection with *E. coli* $tRNA^{Lys,3}$-MA was comparable to that produced by transfection of the HXB2 plasmid which relies on the capture of endogenous mammalian $tRNA^{Lys,3}$ for infectivity. Using a titration of plasmids encoding *E. coli* $tRNA^{Lys,3}$-MA, we found that the amount of infectious virus produced was greater than that following co-transfection with *E. coli* $tRNA^{Lys,3}$ over a wide range of plasmid amounts (Fig. 2C). The increase in the level of complementation was generally 3–5 times greater for *E. coli* $tRNA^{Lys,3}$-MA compared to *E. coli* $tRNA^{Lys,3}$. A mutant *E. coli* $tRNA^{Lys,3}$ with only the A73G mutation alone did not result in increased production of infectious virus (Fig. 2C). Thus, the A73G mutation, made to correspond to the four terminal nucleotides of mammalian $tRNA^{Lys,3}$ within the 3′ terminal 18-nucleotides of *E. coli* $tRNA^{Lys,3}$, did not enhance selection and use of *E. coli* $tRNA^{Lys,3}$ (A73G).

Importance of nucleotides with an anticodon stem for complementation

The major nucleotide differences between *E. coli* $tRNA^{Lys}$-MA and *E. coli* $tRNA^{Lys,3}$ reside in the anticodon stem (Fig. 1A). Previous studies have shown that the anticodon stem–loop of $tRNA^{Lys,3}$ imparts unique properties with respect to interaction with codon–anticodon involved in protein synthesis. Given the fact that the A-loop anticodon interaction has been suggested to resemble a codon–anticodon interaction, it is possible that the nucleotides within this stem–loop facilitate this interaction resulting in the enhanced complementation (Puglisi and Puglisi, 1998). To investigate this possibility, we made a series of mutations in the *E. coli* $tRNA^{Lys,3}$ anticodon stem to determine which would result in enhanced complementation. The first mutation substituted a U–A base pair at nucleotides 27–43 that were G–U base pair in *E. coli* $tRNA^{Lys,3}$ (Fig. 3A). This substitution should result in the introduction of a Ψ–A base pair similar to that seen in mammalian $tRNA^{Lys,3}$. A second mutation was also introduced in which a C–G and A–U base pair at nucleotides 28–42 and 29–41 would give a complete stem sequence of mammalian $tRNA^{Lys,3}$ (Fig. 3A). Analysis of tRNA aminoacylation following transfection revealed that both mutants were aminoacylated similar to that of *E. coli* $tRNA^{Lys,3}$ and *E. coli* $tRNA^{Lys,3}$-MA (data not shown). The capacity of these tRNAs to complement the HIV-1 proviral genome with a

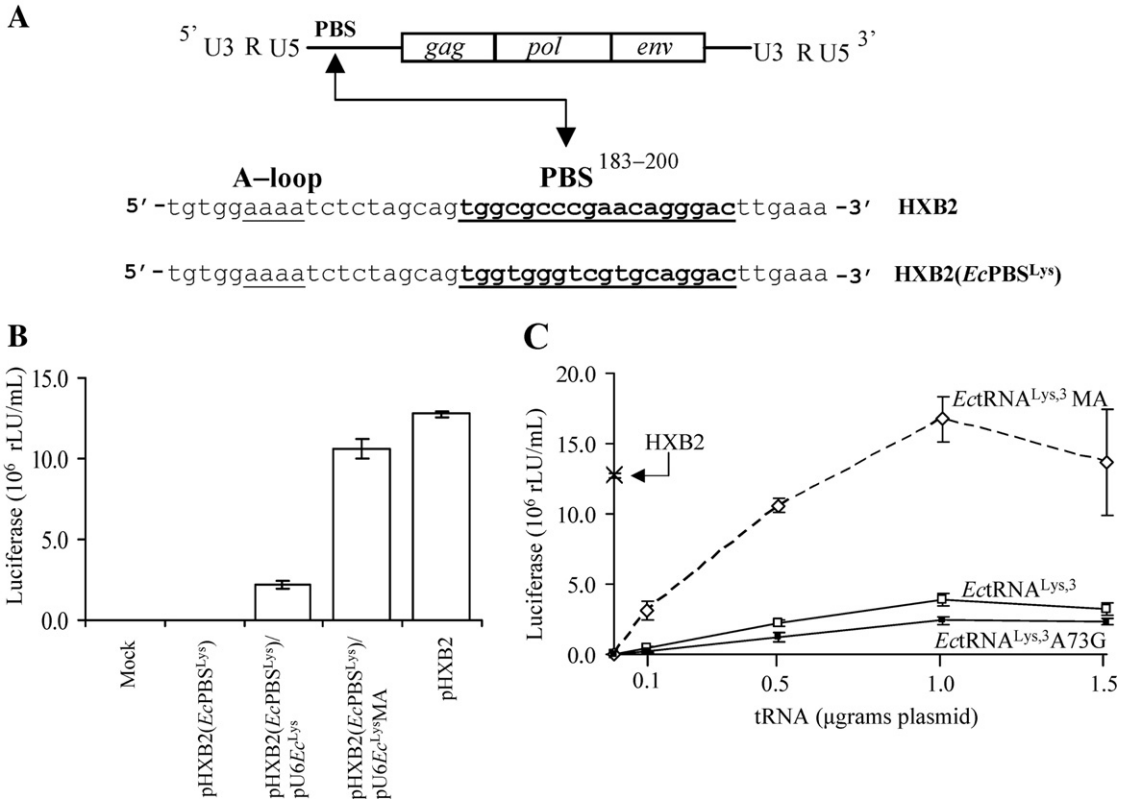

Fig. 2. Complementation of HXB2(*Ec*PBS^{Lys}) with a plasmid that encodes *E. coli* tRNA^{Lys,3}. (A) Diagramatic representation of the proviral U5 region and the corresponding sequence for wild-type HXB2 and mutant HXB2(*Ec*PBS^{Lys}). The underlined sequences correspond to the A-rich region and the PBS. (B) Luciferase activity obtained from infection of JC53-BL cells with viral supernatants collected from co-transfections of 293HEK cells with 500 ng of HIV-1 proviral plasmids in the presence or absence of 500 ng of plasmid encoding the specified tRNA, in relation to wild-type HIV-1 (transfected at 500 ng). The data denote means ± standard deviations derived from three independent transfections. (C) Luciferase activity obtained from infection of JC53-BL cells with viral supernatants obtained from co-transfections of 293HEK cells with 500 ng of proviral plasmids, and tRNA plasmids titrated in at the designated quantities. Luciferase activity, in relative Light Units per mL (rLU/mL), for complementation of plasmid HXB2(*Ec*PBS^{Lys}) with pU6*Ec*^{Lys} is represented by open squares (□); for plasmid HXB2(*Ec*PBS^{Lys}) with pU6*Ec*^{Lys}-MA is represented by open diamonds (◊); for plasmid HXB2(*Ec*PBS^{Lys}) with pU6*Ec*^{Lys}(A73G) is represented with closed squares (■). Wild-type HXB2 (500 ng and no tRNA) is represented by (×).

PBS complementary to *E. coli* tRNA^{Lys,3} was determined. Surprisingly, we found no increase in complementation as the nucleotides within the stem were made to correspond to the stem region of *E. coli* tRNA^{Lys,3}-MA (Fig. 3B). These mutants did complement, but at levels similar to that for the unaltered *E. coli* tRNA^{Lys,3}. Thus, alteration of the nucleotides within the stem of *E. coli* tRNA^{Lys,3} to correspond to that of *E. coli* tRNA^{Lys3}-MA did not impart the enhanced complementation observed with *E. coli* tRNA^{Lys,3}-MA.

Inspection of *E. coli* tRNA^{Lys,3}-MA revealed an additional nucleotide change in the variable loop at nucleotide 44. In *E. coli* tRNA^{Lys,3}, this nucleotide is a U while in mammalian tRNA^{Lys,3} it is a G. To determine the effects of this single nucleotide change, we constructed a mutant *E. coli* tRNA^{Lys,3} in which the U was changed to a G (Fig. 4A). We then characterized the capacity of this tRNA to complement the HIV-1 proviral genome with a PBS complementary to *E. coli* tRNA^{Lys,3}. Analysis revealed that complementation was slightly enhanced with the U44G mutation compared to the complementation with a tRNA containing the stem identical to mammalian tRNA^{Lys,3}, though, the levels of complementation did not reach that for *E. coli* tRNA^{Lys,3}-MA (Fig. 4B). Collectively, the results of these studies demonstrate that the capacity of *E. coli* tRNA^{Lys,3}-MA to complement greater than that observed for *E. coli* tRNA^{Lys,3} resides in the continuity of the nucleotides within the variable loop and the stem of the anticodon stem–loop.

Discussion

In the current study, we have utilized an *in trans* complementation system to ascertain the importance of nucleotides in tRNA^{Lys,3} for selection and use in HIV-1 reverse transcription. Our experiments relied on the use of *E. coli* tRNA^{Lys,3}, which shares many features in common with mammalian tRNA^{Lys,3}. A major difference resides in the 3′ terminal 18-nucleotides, which interact with the HIV-1 PBS, and the nucleotides within the anticodon stem of the tRNA. Although *E. coli* tRNA^{Lys,3} can complement an HIV-1 proviral genome in which the PBS was made complementary to the 3′ terminal 18-nucleotides of this tRNA, we found that the complementation was enhanced if the nucleotides within the variable region and the anticodon stem of *E. coli* tRNA^{Lys,3} were made to correspond to that of mammalian tRNA^{Lys,3} (*E.*

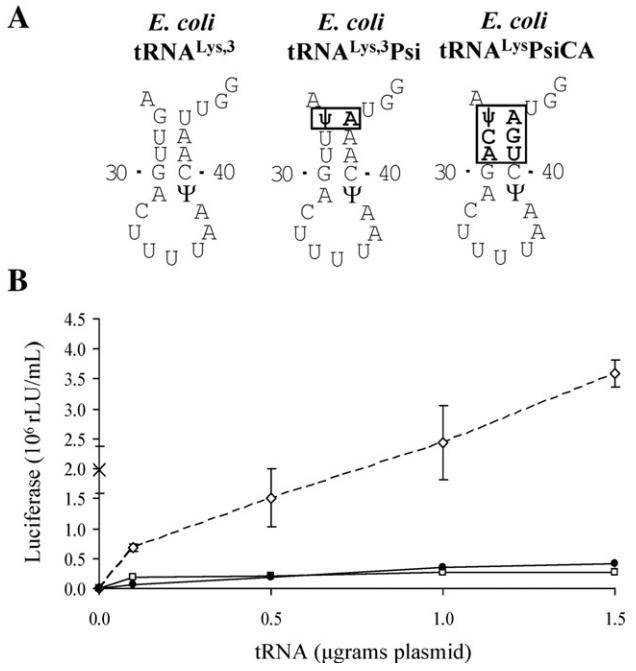

Fig. 3. Complementation of HXB2(*Ec*PBS^{Lys}) with a plasmid that encodes *E. coli* tRNA^{Lys,3}Psi and *E. coli* tRNA^{Lys,3}PsiCA. (A) The anticodon stem–loop sequence of *E. coli* tRNA^{Lys,3}, *E. coli* tRNA^{Lys,3}(Psi), and *E. coli* tRNA^{Lys,3}(PsiCA). The boldface and boxed nucleotides represent base changes made in *E. coli* tRNA^{Lys,3} sequence to match the sequence of mammalian tRNA^{Lys,3}. (B) Luciferase activity obtained from infection of JC53-BL cells with viral supernatants obtained from co-transfections of 293HEK cells with 500 ng of proviral plasmids, and tRNA plasmids titrated in at the designated quantities. Luciferase activity, in relative Light Units per mL (rLU/mL), for complementation of plasmid HXB2(*Ec*PBS^{Lys}) with pU6*Ec*^{Lys}-MA is represented by open diamonds (◊); for plasmid HXB2(*Ec*PBS^{Lys}) with pU6*Ec*^{Lys}(Psi) is represented with closed circles (●); for plasmid HXB2(*Ec*PBS^{Lys}) with pU6*Ec*^{Lys}(PsiCA) is represented with open squares (□). Wild-type HXB2 (500 ng and no tRNA) is represented by (×). The data denote means±standard deviations derived from three independent transfections.

coli tRNA^{Lys,3}-MA). Analysis of the role of individual nucleotides within the variable region and anticodon stem–loop indicated that all changed nucleotides within the variable and anticodon stem–loop were important for the enhanced complementation observed with *E. coli* tRNA^{Lys,3}-MA.

Previous studies have suggested that the RNA structures of *E. coli* tRNA^{Lys,3} and mammalian tRNA^{Lys,3} are similar in the anticodon region (Durant et al., 2005; Sprinzl et al., 1991; Sudarsan et al., 2006; Yarian et al., 2000). Much of this is due to analogous post-transcriptionally modified bases at positions 34 and 37, and a pseudouridine at position 39. Previous studies from our group and others have shown that *E. coli* tRNA^{Lys,3} can be aminoacylated by mammalian lysyl-tRNA synthetase (McClain et al., 1990; McCulley and Morrow, 2006; Stello et al., 1999; Tamura et al., 1992). The major differences between mammalian and *E. coli* tRNA^{Lys,3} reside in the nucleotides sequence in the stem of the anticodon stem–loop. Thus, the results of our studies to mutate nucleotides within the stem to correspond to mammalian tRNA^{Lys,3} were surprising in that they did not result in enhanced complementation by *E. coli* tRNA^{Lys,3}. In fact, the only mutation that generated an increase in complementation, albeit not at the level of *E. coli* tRNA^{Lys,3}-MA, was a mutation in the variable loop. Our results are consistent with the idea that tRNA^{Lys,3} sequence UGAGGG is important for tRNA^{Lys,3} to be effectively selected as the primer for initiation of reverse transcription. Interestingly, this sequence is involved in at least two interactions with the HIV-1 genome (Fig. 5A). In the first, this sequence forms an intermolecular interaction with the U5 region of the HIV-1 viral genome within the tRNA U5 initiation complex (Isel et al., 1993, 1996). To address this issue, we made additional mutations in the HIV-1 proviral genome to compensate for mismatched base pairs as a result of interaction with *E. coli* tRNA^{Lys,3} (Fig. 5B). However, the complementation observed for this virus with *E. coli* tRNA^{Lys,3} was not greater than that found for *E. coli* tRNA^{Lys,3}-MA with the wild-type virus, suggesting that this interaction could not account for the enhanced complementation observed in our study (data not shown). Thus, we do not believe that the lower complementation of *E. coli* tRNA^{Lys,3} compared to tRNA^{Lys,3}-MA was due to the inability to interact with the U5 region. It is possible though that the extensive refolding of tRNA^{Lys,3} required to form the complex between tRNA:viral genome could have

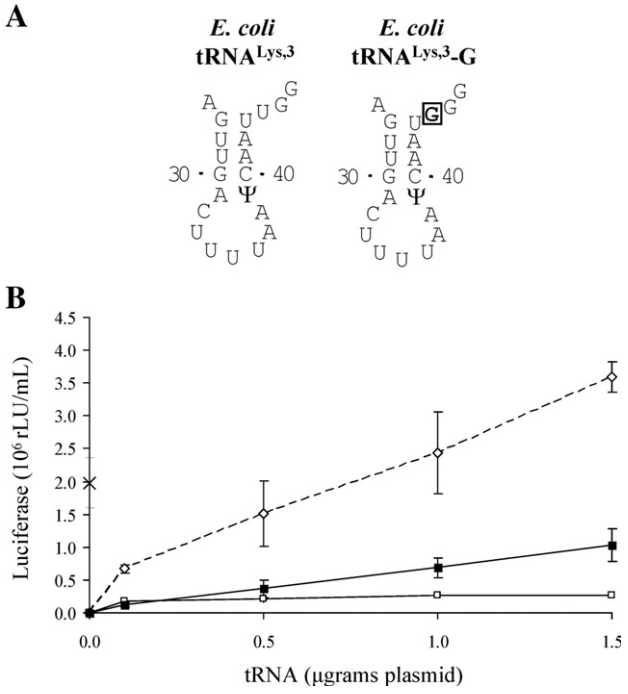

Fig. 4. Complementation of HXB2(*Ec*PBS^{Lys}) with a plasmid that encodes *E. coli* tRNA^{Lys,3}G. (A) The anticodon stem–loop sequence of *E. coli* tRNA^{Lys,3} and *E. coli* tRNA^{Lys,3}(G). The boldface and boxed nucleotide represents base change made in *E. coli* tRNA^{Lys,3} sequence to match the sequence of mammalian tRNA^{Lys,3}. (B) Luciferase activity obtained from infection of JC53-BL cells with viral supernatants obtained from co-transfections of 293HEK cells with 500 ng of proviral plasmids, and tRNA plasmids titrated in at the designated quantities. Luciferase activity, in relative Light Units per mL (rLU/mL), for complementation of plasmid HXB2(*Ec*PBS^{Lys}) with pU6*Ec*^{Lys}-MA is represented by open diamonds (◊); for plasmid HXB2(*Ec*PBS^{Lys}) with pU6*Ec*^{Lys}(G) is represented with closed squares (■); for plasmid HXB2 (*Ec*PBS^{Lys}) with pU6*Ec*^{Lys}(PsiCA) is represented with open squares (□). Wild-type HXB2 (500 ng and no tRNA) is represented by (×). The data denote means±standard deviations derived from three independent transfections.

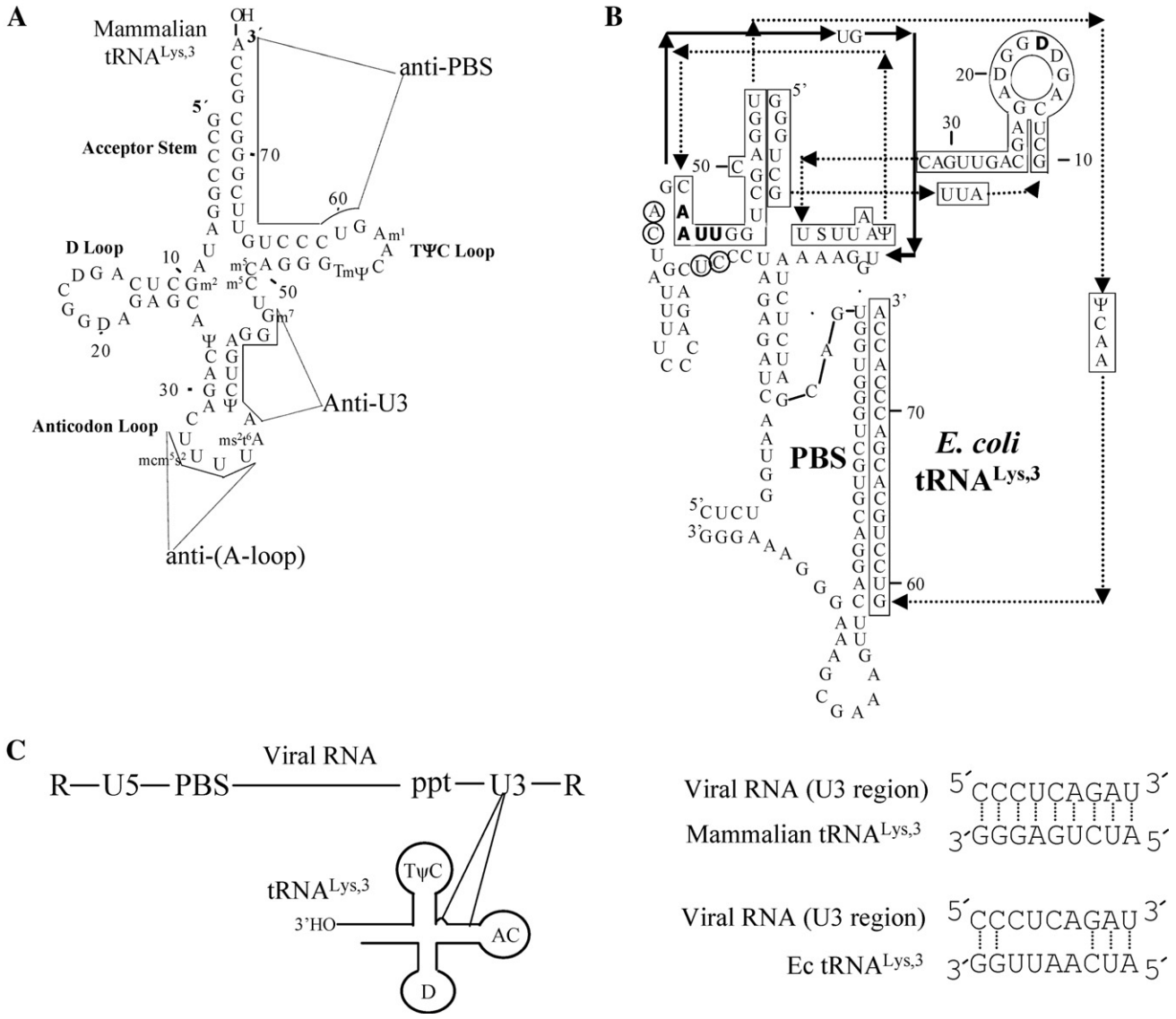

Fig. 5. Mammalian tRNA^{Lys,3} and the corresponding potential interactions with the proviral U3 region. (A) The cloverleaf depiction of mammalian tRNA^{Lys,3} with outlined anti-PBS region, which interacts with the PBS in the viral RNA genome, anti-U3 region, which interacts with the U3 region in the viral RNA genome, and anti-A-loop region, which interacts with the A-rich loop in the viral RNA genome during reverse transcription. (B) Proposed interactions between HIV-1 (HXB2) and *E. coli* tRNA^{Lys,3} based on Isel structure (Isel et al., 1993, 1996). Boxed bases represent *E. coli* tRNA^{Lys,3}. Bolded nucleotides AAUU in *E. coli* tRNA^{Lys,3} proposed to interact with HIV-1 genome in Isel structure; the circled nucleotides in HIV-1 genome were changed to promote Isel structure with *E. coli* tRNA^{Lys,3}. However, HIV-1 genomes with these changes did not show enhanced complementation with *E. coli* tRNA^{Lys,3} (data not shown). The bold D in tRNA^{Lys,3} is in the D loop. (C) Schematic of viral RNA anticodon tRNA^{Lys,3} interaction in the U3 region. The sequence fragment of the viral U3 RNA genome that base pairs with the stem and part of the variable sequence found in the tRNA^{Lys,3}. All nucleotides in mammalian tRNA^{Lys,3} and *E. coli* tRNA^{Lys,3}-MA are complementary to the viral U3 RNA genome sequence, whereas only 5 out of 9 nucleotides are complementary in *E. coli* tRNA^{Lys,3}.

been affected by the nucleotide differences between *E. coli* tRNA^{Lys,3} and tRNA^{Lys,3}-MA. Additional studies will be required to test this possibility.

A second interaction between tRNA^{Lys,3} and the viral genome has been shown from *in vitro* studies where complementarity between the nucleotides in the anticodon stem, part of the variable loop of tRNA^{Lys,3}, and the U3 facilitated the first strand transfer of HIV-1 reverse transcription (Brule et al., 2000). In this *in vitro* system, a several fold enhancement of the strand transfer was observed using an *in vitro* synthesized tRNA that had regions complementary to the regions in U3. Interestingly, the nucleotides within the U3 are highly conserved among all other lentiviruses. There are several nucleotide mismatches between the *E. coli* tRNA^{Lys,3} and the U3 sequence that might reduce the efficiency of strand transfer and hence the production of infectious virus (Fig. 5C). Alteration of the sequence to correspond to tRNA^{Lys,3}-MA provides complete sequence complementarity with the U3 region. However, it is not clear whether this interaction between these specific nucleotides in U3 and tRNA^{Lys,3} is absolutely required. For example, previous studies from this laboratory have identified several tRNAs that can be stably utilized by HIV if A-loop PBS modifications were made (Wakefield et al., 1996; Zhang et al., 1998). One of these viruses, which was engineered

to utilize $tRNA^{His}$ requires an additional change within the A-loop to stably utilize this tRNA for reverse transcription. Brule et al. found that a similar region within the viral RNA genome in U3 is complementary to the sequences within $tRNA^{His}$, but was not the same required for the interaction between U3 and $tRNA^{Lys,3}$ (Brule et al., 2000). Thus, HIV-1 that has been forced to utilize $tRNA^{His}$ by mutations within the PBS and A-loop region might have evolved to alter nucleotides within U3 to facilitate the first strand jump. Additional experiments will be required to delineate the interaction between U3 and $tRNA^{Lys,3}$ during reverse transcription.

Collectively, the results of these studies demonstrate a dynamic relationship between the HIV-1 viral genome and the host during acquisition of the primer utilized for virus replication. The results of our studies are consistent with the concept that HIV-1 has evolved to efficiently select and use $tRNA^{Lys,3}$ as the preferred primer through in part maximization of tRNA:genome RNA interactions. To date, the 3′ terminal 18-nucleotides, TΨC loop, and anticodon stem–loop region of $tRNA^{Lys,3}$ have been shown to be involved with the interaction (Fig. 5A). Further studies using this unique *in trans* complementation system will allow elucidation of the interactions between the $tRNA^{Lys,3}$ and the viral genome required for the preferential selection of $tRNA^{Lys,3}$.

Materials and methods

Tissue culture

The 293HEK and JC53-BL cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (Gibco/BRL, Gaithersburg, MD.). Cell cultures were maintained in 37 °C incubator supplemented with 5% CO_2.

Proviral and tRNA plasmids

The proviral plasmid encoding the full length HXB2 with a PBS altered to be complementary to the 3′-terminal 18-nucleotides of *E. coli* $tRNA^{Lys,3}$ and the *E. coli* $tRNA^{Lys}$ gene was constructed previously (McCulley and Morrow, 2006). The *E. coli* $tRNA^{Lys,3}$(A73G), *E. coli* $tRNA^{Lys,3}$(MA), *E. coli* $tRNA^{Lys,3}$(PsiCA), *E. coli* $tRNA^{Lys,3}$(Psi), and *E. coli* $tRNA^{Lys,3}$(G) were prepared by PCR extension with the use of the following primers: (forward A73G) GCAGGGCTCGAGGTCCGGGTCGTTAGCTCAGTTGGTAGAGCAGTTGACTTTTAATCAATTGGTCGCAGG3′, (reverse A73G) GCGGACGAAGCTTCCAAAAAACGGGTCGTGCAGGACTTGAACCTGCGACCAATTGATTAAAAGTCAA3′; (forward MA) 5′GCAGGGCTCGAGGTCCGGGTCGTTAGCTCAGTCGGTAGAGCATCAGACTTTTAATCTGAGGGTCGCAGG3′, (reverse MA) 5′GCGGACGAAGCTTCCAAAAAATGGGTCGTGCAGGACTTGAACCTGCGACCCTCAGATTAAAAGTCTG3′; (forward PsiCA) 5′GCAGGGCTCGAGGTCCGGGTCGTTAGCTCAGTTGGTAGAGCATCAGACTTTTAATCTGATGGTCGCAGG3′, (reverse PsiCA) 5′GCGGACGAAGCTTCCAAAAAATGGGTCGTGCAGGACTTGAACCTGCGACCATCAGATTAAAAGTCTGATG3′; (forward Psi) 5′GCAGGGCTCGAGGTCCGGGTCGTTAGCTCAGTTGGTAGAGCATTTGACTTTTAATCAAATGGTCGCAGG3′, (reverse Psi) 5′GCGGACGAAGCTTCCAAAAAATGGGTCGTGCAGGACTTGAACCTGCGACCATTTGATTAAAAGTCAAATG3′; (forward U43G) 5′GCAGGGCTCGAGGTCCGGGTCGTTAGCTCAGTTGGTAGAGCAGTTGACTTTTAATCAATGGGTCGCAGG3′, (reverse U43G) 5′GCGGACGAAGCTTCCAAAAAATGGGTCGTGCAGGACTTGAACCTGCGACCCCATTGATTAAAAGTCAACTG3′. The PCR products were TA cloned into pGEM T-Easy vector (Promega, Madison, WI), and the resultant TA clones were digested using *Xho*I and *Hin*dIII restriction enzymes in order to release the *E. coli* $tRNA^{Lys}$ constructs (approximately 100-bp). Subsequently, the *E. coli* $tRNA^{Lys}$ constructs were ligated into an LS9 plasmid upstream of a polymerase III promoter using the *Xho*I and *Hin*dIII restriction sites (Kelly and Morrow, 2003, 2005; Kelly et al., 2003). The end products resulted in plasmids labeled pU6Ec^{Lys}(A73G), pU6Ec^{Lys}(MA), pU6Ec^{Lys}(PsiCA), pU6Ec^{Lys}(Psi), and pU6Ec^{Lys}(U43G). All plasmids were screened with enzyme restriction and verified by DNA sequencing.

Co-transfections

Complementation of HIV-1 proviral mutants was performed in a previously described manner (McCulley and Morrow, 2006). Briefly, 293HEK cells were co-transfected with tRNA-carrying plasmids and HIV-1 proviral mutants 24 h after seeding in 6-well plates. Transfections were achieved using a calcium-phosphate method previously described (Jordan et al., 1996). The cells were co-transfected with 500 ng of proviral plasmid and 100 ng, 500 ng, 1000 ng, and 1500 ng of tRNA-carrying plasmid. Mock and control (proviral plasmids alone, or tRNA plasmids alone) transfections were also included. Approximately 7 h after co-transfection, the cells were washed once with 1× PBS and replenished with fresh media. Approximately 48 h post-transfection, supernatants were collected, centrifuged at 3000×*g*, and serially diluted for infection of JC53-BL cells.

JC53-BL Infections

Supernatants collected from co-transfections were used in JC53-BL reporter assay in order to determine infectious viral units from luciferase activity (Wei et al., 2002). JC53-BL assay was performed as described previously (McCulley and Morrow, 2006). Briefly, supernatants were diluted 1:3 in DMEM supplemented with 2% FBS, followed by two sequential 1:5 dilutions, and used to infect JC53-BL cells that were seeded 24 h pre-infection. JC53-BL cells with the added supernatants were incubated for 2 h in a 37 °C incubator supplemented with 5% CO_2. After 2 h, DMEM supplemented with 10% FBS was added to each well and the cells were incubated for additional 48 h. To determine luciferase activity, cells were lysed using M-PER Mammalian Protein Extraction Reagent (PIERCE,

Rockford, IL) and approximately 20 μL of each sample was transferred to a microplate. Reporter Lysis Buffer (Promega, Madison, WI) was added to each sample in the microplate and the light intensity was measured using a Tropix TR717 Microplate Luminometer (Applied Biosystems, Foster City, CA). Wells that contained lysed samples from uninfected cells represented background and the luciferase activity obtained from these wells was subtracted from all other samples. Luciferase activity for pHXB2(*Ec*PBS^{Lys}), without the complementing tRNA, was set as background also. The difference obtained from subtracting luciferase activity values of uninfected samples from luciferase activity values of pHXB2 (*Ec*PBS^{Lys}), without the complementing tRNA, was subtracted from all complementation samples. The luciferase activity values of two dilutions per sample were averaged. Relative Light Units (rLU) per mL were calculated by dividing the luciferase activity values by their corresponding dilutions.

RNA isolation and tRNA analysis

293HEK cells were transfected with pU6*Ec*^{Lys}, pU6*Ec*^{Lys} (A73G), pU6*Ec*^{Lys}(MA), pU6*Ec*^{Lys}(PsiCA), pU6*Ec*^{Lys}(Psi), and pU6*Ec*^{Lys}(U43G) using calcium phosphate via a previously described method (Jordan et al., 1996). Aminoacyl tRNAs were collected approximately 48 h post-transfection. Collection and isolation of aminoacylated tRNAs were performed as previously described (Kelly and Morrow, 2003, 2005; Kelly et al., 2003; McCulley and Morrow, 2006). Aminoacyl tRNAs were also isolated from mock-transfected 293HEK cells. All *E. coli* tRNA^{Lys} molecules were detected using a [γ-^{32}P] ATP kinased oligo, 5′GGTCGTGCAGGATTCGAACCTGCGACCAA3′, that was labeled with the use of Ready-to-go T4 polynucleotide kinase (Amersham Pharmacia Biotech, Piscataway, NJ). Aminoacyl tRNAs were separated based on charge using an acidic polyacrylamide gel and analyzed using Northern blotting (Kelly and Morrow, 2003, 2005; Kelly et al., 2003; McCulley and Morrow, 2006). X-ray film was exposed to the probed membranes and developed using the SRX-101A developer (Konica, Wayne, NJ).

Acknowledgments

We thank members of the Morrow Laboratory for helpful suggestions. Adrienne Ellis is acknowledged for preparation of the manuscript. AM was supported by training grant AI07493. DNA sequencing was carried out by the UAB CFAR DNA Sequencing Core (AI 27767). CDM acknowledges insights from MAR. This work was supported by a grant from the NIH to CDM (AI34749).

References

Beerens, N., Groot, F., Berkhout, B., 2001. Initiation of HIV-1 reverse transcription is regulated by a primer activation signal. J. Biol. Chem. 276 (33), 31247–31256.

Brule, F., Bec, G., Keith, G., Le Grice, S.F.J., Roques, B.P., Ehresmann, B., Ehresmann, C., Marquet, R., 2000. *In vitro* evidence for the interaction of tRNA$_3^{Lys}$ with U3 during the first strand transfer of HIV-1 reverse transcription. Nucleic Acids Res. 28 (2), 634–640.

Cen, S., Javanbakht, H., Kim, S., Shiba, K., Craven, R.C., Rein, A., Ewalt, K.L., Schimmel, P., Musier-Forsyth, K., Kleiman, L., 2002. Retrovirus-specific packaging of aminoacyl-tRNA synthetases with cognate primer tRNAs. J. Virol. 76, 13111–13115.

Das, A.T., Klaver, B., Berkhout, B., 1995. Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural tRNA^{Lys,3}. J. Virol. 69 (5), 3090–3097.

Durant, P.C., Bajji, A.C., Sundaram, M., Kumar, R.K., Davis, D.R., 2005. Structural effects of hypermodified nucleosides in the *Escherichia coli* and human tRNALys anticodon loop: the effect of nucleosides s^{2}U, mcm^{5}U, mcm^{5}s^{2}U, mnm^{5}s^{2}U, t^{6}A, and ms^{2}t^{6}A. Biochemistry 44, 8078–8089.

Huang, Y., Shalom, A., Li, Z., Wang, J., Mak, J., Wainberg, M.A., Kleiman, L., 1996. Effects of modifying the tRNA^{Lys,3} anticodon on the initiation of human immunodeficiency virus type 1 reverse transcription. J. Virol. 70, 4700–4706.

Isel, C., Marquet, R., Keith, G., Ehresmann, C., Ehresmann, B., 1993. Modified nucleotides of tRNA^{Lys3} modulate primer/template loop–loop interaction in the initiation complex of HIV-1 reverse transcription. J. Biol. Chem. 268 (34), 25269–25272.

Isel, C., Lanchy, J.M., Le Grice, S.F., Ehresmann, C., Ehresmann, B., Marquet, R., 1996. Specific initiation and switch to elongation of human immunodeficiency virus type 1 reverse transcription require the post-transcriptional modifications of primer tRNA3Lys. EMBO J. 15, 917–924.

Javanbakht, H., Halwani, R., Cen, S., Saadatmand, J., Musier-Forsyth, K., Gottlinger, H., Kleiman, L., 2003. The interaction between HIV-1 *Gag* and human lysyl-tRNA synthetase during viral assembly. J. Biol. Chem. 278 (30), 27644–27651.

Jordan, M., Schallhorn, A., Wurm, F.M., 1996. Transfecting mammalian cells: optimization of critical parameter affecting calcium-phosphate precipitate formation. Nucleic Acids Res. 24 (4), 596–601.

Kang, S.-M., Morrow, C.D., 1999. Genetic analysis of a unique human immunodeficiency virus type 1 (HIV-1) with a primer binding site complementary to tRNA^{Met} supports a role for U5-PBS stem–loop RNA structures in initiation of HIV-1 reverse transcription. J. Virol. 73, 1818–1827.

Kang, S.-M., Zhang, Z., Morrow, C.D., 1997. Identification of a sequence within U5 required for human immunodeficiency virus type 1 to stably maintain a primer binding site complementary to tRNA^{Met}. J. Virol. 71 (1), 207–217.

Kang, S.-M., Zhang, Z., Morrow, C.D., 1999. Identification of a human immunodeficiency virus type 1 that stably uses tRNA^{Lys1,2} rather than tRNA^{Lys,3} for initiation of reverse transcription. Virology 257, 95–105.

Kelly, N.J., Morrow, C.D., 2003. Yeast tRNA^{Phe} expressed in human cells can be selected by HIV-1 for use as a reverse transcription primer. Virology 313, 354–363.

Kelly, N.J., Morrow, C.D., 2005. Structural elements of the tRNA Tpsi C loop critical for nucleocytoplasmic transport are important for human immunodeficiency virus type 1 primer selection. J. Virol. 79, 6532–6539.

Kelly, N.J., Palmer, M.T., Morrow, C.D., 2003. Selection of retroviral reverse transcription primer is coordinated with tRNA biogenesis. J. Virol. 77, 8695–8701.

Li, X., Mak, J., Arts, E.J., Gu, Z., Kleiman, L., Wainberg, M.A., Parniak, M.A., 1994. Effects of alterations of primer-binding site sequences on human immunodeficiency virus type 1 replication. J. Virol. 68 (10), 6198–6206.

Mak, J., Khorchid, A., Cao, Q., Huang, Y., Lowy, I., Parniak, M.A., Prasad, V.R., Wainberg, M.A., Kleiman, L., 1997. Effects of mutations in Pr160$^{gag\text{-}pol}$ upon tRNA^{Lys,3} and Pr160$^{gag\text{-}pol}$ incorporation into HIV-1. J. Mol. Biol. 265, 419–431.

Marquet, R., Isel, C., Ehresmann, C., Ehresmann, B., 1995. tRNAs as primer of reverse transcriptases. Biochimie 77, 113–124.

McClain, W.H., Foss, K., Jenkins, R.A., Schneider, J., 1990. Nucleotides that determine *Escherichia coli* tRNA^{Arg} and tRNA^{Lys} acceptor identities revealed by analyses of mutant opal and amber suppressor tRNAs. Proc. Natl. Acad. Sci. U.S.A. 87, 9260–9264.

McCulley, A., Morrow, C.D., 2006. Complementation of human immunodeficiency virus type 1 replication by intracellular selection of *Escherichia coli* tRNA^{Lys,3} supplied *in trans*. J. Virol. 80 (19), 9641–9650.

Puglisi, E.V., Puglisi, J.D., 1998. HIV-1 A-rich RNA loop mimics the tRNA anticodon structure. Nat. Struct. Biol. 5 (12), 1033–1036.

Shiba, K., Stello, T., Motegi, H., Noda, T., Musier-Forsyth, K., Schimmel, P., 1997. Human lysyl-tRNA synthetase accepts nucleotide 73 variants and rescues *Escherichia coli* double-defective mutant. J. Biol. Chem. 272 (36), 22809–22816.

Sprinzl, M., Dank, N., Nock, S., Schon, A., 1991. Compilation of tRNA sequences and sequences of tRNA genes. Nucleic Acids Res. 19, 2127–2171.

Stello, T., Hong, M., Musier-Forsyth, K., 1999. Efficient aminoacylation of $tRNA^{Lys,3}$ by human lysyl-tRNA synthetase is dependent on covalent continuity between the acceptor stem and the anticodon domain. Nucleic Acids Res. 27, 4823–4829.

Sudarsan, N., Hammond, M.C., Block, K.F., Welz, R., Barrick, J.E., Roth, A., Breaker, R.R., 2006. Tandem riboswitch architectures exhibit complex gene control functions. Science 314 (5797), 300–304.

Tamura, K., Himeno, H., Asahara, H., Hasegawa, T., Shimizu, M., 1992. *In vitro* study of *E. coli* $tRNA^{Arg}$ and $tRNA^{Lys}$ identity elements. Nucleic Acids Res. 20, 2335–2339.

Wakefield, J.K., Wolf, A.G., Morrow, C.D., 1995. Human immunodeficiency virus type 1 can use different tRNAs as primers for reverse transcription but selectively maintains a primer binding site complementary to $tRNA^{Lys,3}$. J. Virol. 69, 6021–6029.

Wakefield, J.K., Kang, S.-M., Morrow, C.D., 1996. Construction of a type 1 human immunodeficiency virus that maintains a primer binding site complementary to $tRNA^{His}$. J. Virol. 70, 966–975.

Wei, X., Decker, J.M., Liu, H.M., Zhang, Z., Arani, R.B., Kilby, J.M., Saag, M.S., Wu, X., Shaw, G.M., Kappes, J.C., 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob. Agents Chemother. 46 (6), 1896–1905.

Yarian, C., Marszlek, M., Sochacka, E., Malkiewicz, A., Guenther, R., Miskiewicz, A., Agris, P.F., 2000. Modified nucleoside dependent Watson-Crick and Wobble codon binding by $tRNA^{Lys}_{UUU}$ species. Biochemistry 39, 13390–13395.

Yu, Q., Morrow, C.D., 2000. Essential regions of the tRNA primer required for HIV-1 infectivity. Nucleic Acids Res. 28, 4783–4789.

Yu, Q., Morrow, C.D., 2001. Identification of critical elements in the tRNA acceptor stem and TYC loop necessary for human immunodeficiency virus type 1 infectivity. J. Virol. 75, 4902–4906.

Zhang, Z., Kang, S.-M., Li, Y., Morrow, C.D., 1998. Genetic analysis of the U5-PBS of a novel HIV-1 reveals multiple interactions between the tRNA and RNA genome required for initiation of reverse transcription. RNA 4, 394–406.